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The F_1F_0-ATPase of *Bifidobacterium animalis* is involved in bile tolerance

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Summary

Adaptation and tolerance to bile stress are important factors for the survival of bifidobacteria in the intestinal tract. *Bifidobacterium animalis* is a probiotic microorganism which has been largely applied in fermented dairy foods due to its technological properties and its health-promoting effects for humans. The effect of the presence of bile on the activity and expression of F_1F_0-ATPase, the pool of ATP and the intracellular pH of *B. animalis* IPLA 4549 and its mutant with acquired resistance to bile *B. animalis* 4549dOx was determined. The bile-resistant mutant tolerated the acid pH better than its parent strain. Bile induced the expression of the F_1F_0-ATPase and increased the membrane-bound H^+-ATPase activity, in both parent and mutant strains. In acidic conditions (pH 5.0), the expression and the activity of this enzyme were higher in the mutant than in the parent strain when cells were grown in the absence of bile. Total ATP content was higher for the mutant in the absence of bile, whereas the presence of bile induced a decrease of intracellular ATP levels, which was much more pronounced for the parent strain. At pH 4.0, and independently on the presence or absence of bile, the mutant showed a higher intracellular pH than its parent strain. These findings suggest that the bile-adapted *B. animalis* strain is able to tolerate bile by increasing the intracellular ATP reserve, and by inducing proton pumping by the F_1F_0-ATPase, therefore tightly regulating the internal pH, and provide a link between the physiological state of the cell and the response to bile.

Introduction

Bifidobacteria are anaerobic, Gram-positive, irregular fermentative rod-shaped bacteria. The G+C content of its DNA ranges between 55% and 64%, being thus included in the *Actinobacteria* class (Scardovi, 1986). They are important components of the gastrointestinal microbiota, in which they may be present at concentrations of 10^9–10^{11} cells per gram of faeces, representing up to 91% of microbial gut population in the colon during the early stage of life (Langendijk *et al.*, 1995; Harmsen *et al.*, 2000). Increasing interest has arisen during the past two decades on the potential probiotic role of these microorganisms in human well-being, leading to a deeper understanding of their physiology, metabolism and genetics (Meile *et al.*, 2001; Schell *et al.*, 2002; Isolauri *et al.*, 2004; Klijn *et al.*, 2005). Particularly, the appropriate technological properties of *Bifidobacterium animalis* ssp. *lactis* have caused some strains of this species to be largely applied in functional foods (Masco *et al.*, 2005).

After ingestion, bifidobacteria meet several biological barriers, the most important being the gastric acidity and the bile in the intestine. Bile salts are detergent-like biological compounds that disorganize the lipid bilayer structure of cellular membranes, induce protein misfolding and cause oxidative damage to the DNA (Bernstein *et al.*, 1999). Therefore, the adaptation of bifidobacteria to bile salts, present in the colon at concentrations usually below 5 mM (Hofmann, 1999), is critical for the colonization of the gut.

On the other hand, H^+-ATPases, also designed as F_1F_0-ATPases or ATP synthases, are membrane-embedded enzymes which are involved in crucial biological functions (Boyer, 2002). For many microbial inhabitants of the gastrointestinal tract, the F_1F_0-ATPase is an important element in the response and tolerance to low pH (Arikado *et al.*, 1999; Kullen and Klaenhammer, 1999; Kuhnert *et al.*, 2004; Matsumoto *et al.*, 2004). In bacteria lacking a respiratory chain, such as *Bifidobacterium*, its role is to create a proton gradient at the expense of ATP consumption; by pumping protons from the cytoplasm to the exterior, this enzyme functions to assist in maintaining physiologically relevant internal pH values in acidic environments (Cotter and Hill, 2003). In *B. animalis* ssp. *lactis* DSM10140 (formerly *Bifidobacterium lactis* DSM10140; Ventura and Zink, 2002; Masco *et al.*, 2004), the F_1F_0-ATPase is an important element in the response to acidic conditions (Ventura *et al.*, 2004a). Furthermore, this enzyme has been proposed to be related with some

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stress stimuli. In eukaryotic cells, the α-subunit of the mitochondrial F_1-ATPase has been shown to be a heat-shock protein in *Drosophila hydei* (Luis *et al*., 1990), and to respond to salt stress in barley (Fukuda *et al*., 2004). In bacteria, the ATP synthase is involved in the response to protonophoric hop compounds in *Lactobacillus brevis* (Sakamoto *et al*., 2002), and its α-subunit was found to be upregulated in *Escherichia coli* during adaptation to glucose-limited conditions (Wick *et al*., 2001). Moreover, in the last years, high-throughput genomic and proteomic analysis has allowed the establishment of putative new physiological roles for this enzyme in some Gram-positive bacteria. Its γ-chain was overproduced in *Propionibacterium freudenreichii* as a response to bile (Leverrier *et al*., 2003). A DNA microarray based identification of bile-responsive genes in *Lactobacillus plantarum* showed that the F_1F_0-ATPase potentially contributes to the maintenance of proton gradients during bile stress (Bron *et al*., 2006). The theoretical role for the involvement of this enzyme in bile response should be to maintain the proton motive force and the internal pH at viable levels. However, to the best of our knowledge, there are not detailed studies that effectively prove a link between the F_1F_0-ATPase function and bile response and tolerance.

We have previously obtained a bile salt resistant mutant from *B. animalis* ssp. *lactis* IPLA 4549 (named as *B. animalis* 4549dOx) by progressive adaptation to increasing concentrations of ox bile. The bile resistance phenotype was stable and several physiological properties, such as the adhesion ability, carbohydrate fermentation patterns and tolerance to low pH, were modified in the mutant with respect to the parent strain (Noriega *et al*., 2004; Gueimonde *et al*., 2005; Ruas-Madiedo *et al*., 2005). Remarkably, the bile-resistant mutant also showed an enhanced survival at acidic pH. This fact prompted us to the present work, in which we demonstrated the involvement of the membrane H^+-ATPase in the tolerance to bile.

Results

Growth in the presence of bile salts

Our first approach was the determination of sublethal bile concentrations for our strains. *Bifidobacterium animalis* IPLA 4549 and its bile-resistant mutant *B. animalis* 4549dOx were grown in batch culture. Growth rates were higher for the parent strain than for the mutant in the absence of bile. Furthermore, when bile was added to the medium, the growth rates of the parent strain decreased much more sharply than that of the mutant (Fig. 1). We did not detect significant growth for the parent strain at 10 g l^{-1} bile. Therefore, for most of the experiments performed in this work, we have used 3 g l^{-1} bile salts (approximately 6 mM).

Tolerance to low pH under energized conditions

The capacity to survive in acidic conditions (pH 2.0) was tested for the parent strain and the bile-resistant mutant, in the absence and presence of glucose (Fig. 2). The mutant displayed considerably higher survival than the parent strain through the exposure to low pH both in the absence and presence of glucose. However, the energization of the cells with glucose considerably increased the survival of both strains (viable counts were between 2 and 3 log units higher), indicating that the tolerance to acid is probably an energy-dependent mechanism.

Western blot analysis

In preliminary experiments, two different antisera against the β-subunit of the H^+-ATPase of *E. coli* (a mouse antiserum provided by Dr Gabriele Deckers-Hebestreit and a rabbit antiserum provided by Dr Stanley D. Dunn) were

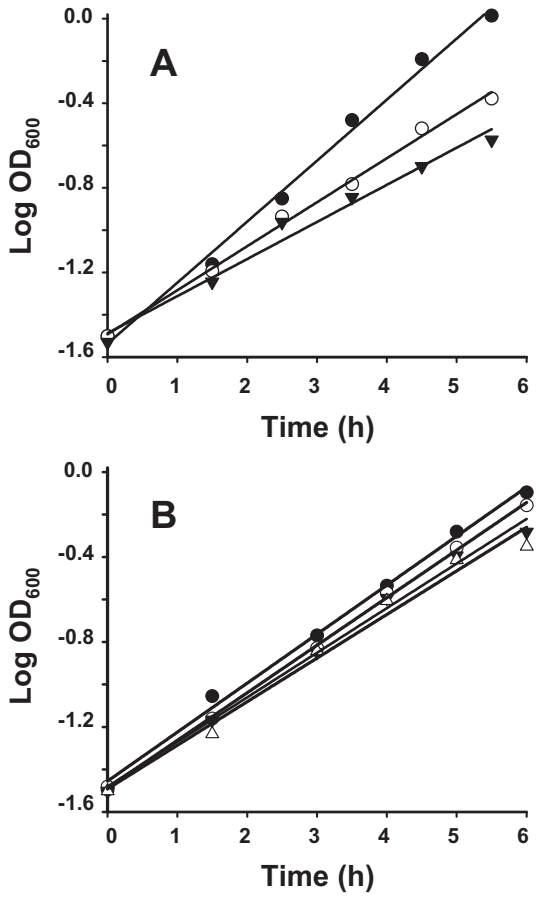

Fig. 1. Growth of *Bifidobacterium animalis* IPLA 4549 (A) and its bile-resistant mutant *B. animalis* 4549dOx (B) in the presence of increasing concentrations of bile. Growth was monitored by OD_{600} measurements in the absence of bile salts (●), with 3 g l^{-1} bile salts (○), 6 g l^{-1} bile salts (▼) or 10 g l^{-1} bile salts (△). Each point represents the mean value of two independent experiments.

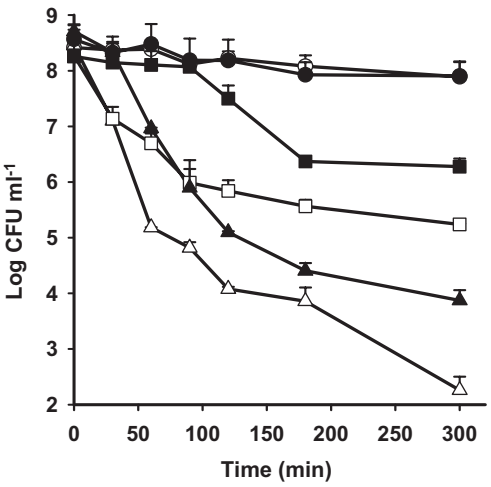

Fig. 2. Susceptibility to acid (pH 2.0) of the parent strain (open symbols) and its bile-resistant mutant (filled symbols). The circles represent survival after exposure to a control solution (NaCl 5 g l^{-1}, pH 5.4), and triangles and squares represent survival after exposure to pH 2.0 in the absence and presence of 25 mM glucose respectively. Error bars represent standard deviations of triplicate experiments.

used. With both of them, a coincident band was detected with similar molecular weight to the *E. coli* β-subunit. However, the signal was stronger with the anti-rabbit antibodies that were raised against the β-subunit extracted from SDS-PAGE gels of purified *E. coli* F_1-ATPase (S.D. Dunn, pers. comm.). Therefore, this antiserum was selected for Western blot analysis (Fig. 3). Our results strongly suggested the F_1F_0-type nature of the ATPase of *B. animalis*. In addition, the intensity of the bands obtained for the parent strain were slightly higher at pH 5.0 than at pH 6.0, but the signal considerably increased at both pHs when bile was present in the medium. In the mutant, an abrupt increase of the intensity of the band was obtained in the presence of bile at pH 6.0, whereas at pH 5.0 the band intensity was similar in the absence and presence of bile, with levels comparable to that obtained at pH 6.0 in the presence of bile.

Membrane ATPase activity and effect of inhibitors and bile

Firstly, we determined the optimal pH for the ATPase activity. Membranes of both *B. animalis* IPLA 4549 and its bile-resistant mutant showed maximum activity when the pH in the reaction buffer was around 5.25 (Fig. 4A, data not shown for the mutant). Therefore, pH 5.25 was chosen for further experiments.

To characterize the type of ATPase present in the membrane of *B. animalis*, and to determine the activity the F_1F_0-ATPase is responsible for, the effect of several inhibitors on the ATPase activity was studied for membranes of the parent and the mutant strains, grown under different pH values and bile concentrations (Fig. 4B). ATPase activity was moderately affected by the well-known inhibitor of ABC-transporters and P-type ATPases *ortho*-vanadate (Yokota *et al*., 2000) and by the V-type inhibitor K_2NO_3 (Kakinuma and Igarashi, 1994). However, the F_1F_0-type inhibitor *N,N′*-dicyclohexylcarbodiimide (DCCD) (Solioz, 1984) was the strongest effector of the ATPase activity. This result indicated that the F-type ATPase is responsible for the majority of the ATPase activity found in the membrane of *B. animalis*. The deduced F_1F_0-ATPase activity, calculated as the difference between the total ATPase activity (black bars) and the ATPase activity in the presence of DCCD (white bars), correlated well with the intensities of the bands obtained in the Western blots under the same conditions (correlation coefficient 0.981), supporting the results of the immunoblotting. Thus, the presence of bile promoted an increase of F_1F_0-ATPase activity on the parent strain at pH 6.0 and pH 5.0, and on the mutant at pH 6.0, whereas the levels of the activity remained always high in the mutant at pH 5.0, independently on the presence or absence of bile.

Intracellular ATP concentration

To further study the physiological state of the cells, the ATP levels in *B. animalis* were determined in a luciferase-driven assay (Fig. 5). For parent and mutant grown in the absence of bile, the intracellular ATP content was always higher at pH 6.0 than at pH 5.0. Also, under the same pH conditions, the mutant always maintained higher ATP levels. Remarkably, when bile was present in the growth

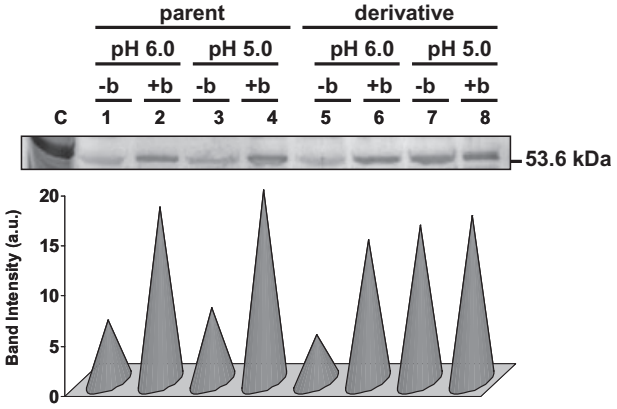

Fig. 3. Western blot analysis of membranes of *Bifidobacterium animalis* and *Escherichia coli*. Parent strain (lanes 1–4) and mutant (lanes 5–8) were grown up to OD_{600} 0.6 under different pH conditions, in the absence (–b) or presence (+b) of 3 g l^{-1} ox bile. Proteins were transferred to a PVDF membrane and developed with antisera against the β-subunit of the F_1F_0-ATPase of *E. coli*. Membranes from *E. coli* (lane c) were used as positive control. The molecular mass of ovalbumin is indicated. The graphic below represents the intensities of the bands measured with the ImageMaster 2D Platinum software from Amersham Biosciences and expressed in arbitrary units. This band profile was reproduced in three independent experiments with three different batches of membrane vesicles.

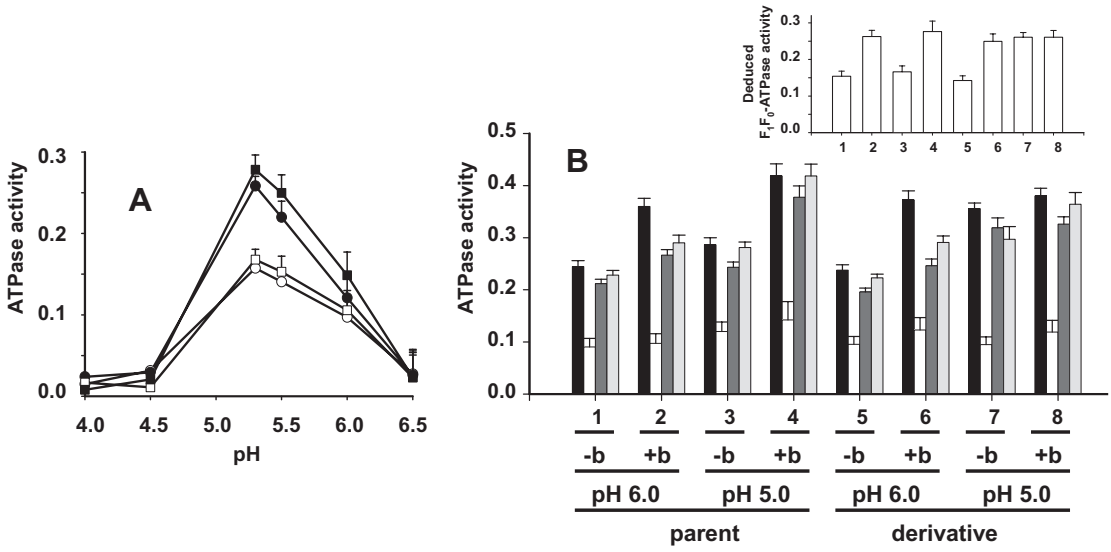

Fig. 4. A. pH profile of the H^+-ATPase activity in membrane vesicles of the parent strain grown at pH 6.0 (circles) or pH 5.0 (squares) in the absence (open symbols) or presence (closed symbols) of 3 g l^{-1} bile. The activity was calculated as the difference between the total ATPase activity and the ATPase activity in the presence of DCCD.
B. Effects of inhibitors on the membrane ATPase activity of *Bifidobacterium animalis*. Parent strain (1–4) and mutant (5–8) were grown up to OD_{600} 0.6 under different pH conditions, in the absence (–b) or presence (+b) of 3 g l^{-1} ox bile. The ATPase activity was measured at pH 5.25 in the presence of 0.2 mM DCCD (white bars), 0.2 mM *ortho*-vanadate (dark grey bars) or 25 mM nitrate (light grey bars). The total ATPase activity without any inhibitor (solid bars) was measured as a control. Error bars represent standard deviations experiments with three different batches of membrane vesicles. The activity is expressed as units per mg of protein. The H^+-ATPase activity, calculated as the difference between the total ATPase activity (black bars) and the ATPase activity in the presence of DCCD (white bars), is represented in the inset.

medium, we observed an abrupt ATP depletion in the parent strain at both pHs assayed, whereas this decrease was much more moderate for the mutant under the same bile concentration. Only when bile was present at concentrations as high as 10 g l^{-1}, the mutant reached levels of ATP as low as the parent strain with 3 g l^{-1} bile.

Intracellular pH determination

Measurements of intracellular pH (pH_{in}) values at three different external pHs (pH_{out}) were recorded for the parent and the bile-resistant mutant strains in the absence and presence of bile (Fig. 6). As expected, the higher pH_{out}, the higher pH_{in} was observed. The presence of bile in the assay buffer reduced the pH_{in} in all cases. It is important to remark that parent and mutant were able to maintain the pH_{in} above 6 when the pH_{out} was 6.0 or 5.0. However, the largest differences between both strains were observed at pH_{out} 4.0, for which the mutant was able to maintain a pH_{in} much higher than the parent. At this low pH and in the absence of bile, pH_{in} was 5.32 for the parent and 5.89 for the mutant, whereas in the presence of bile, the parent strain had a pH_{in} of 5.06, and the mutant maintained a pH_{in} of 5.80. This indicated that under bile and/or acidic pH challenge, the mutant has an enhanced capacity to keep the pH_{in} close to a physiologically relevant pH.

Discussion

In recent years, it is becoming increasingly obvious that many stress response systems overlap and are closely related (O'Sullivan and Condon, 1997; Moskvina *et al*., 1999; Rallu *et al*., 2000; Wright, 2004). To unravel the molecular mechanisms leading to these processes, molecular techniques for disrupting genes and controlling gene expression have been extensively used. Specifically,

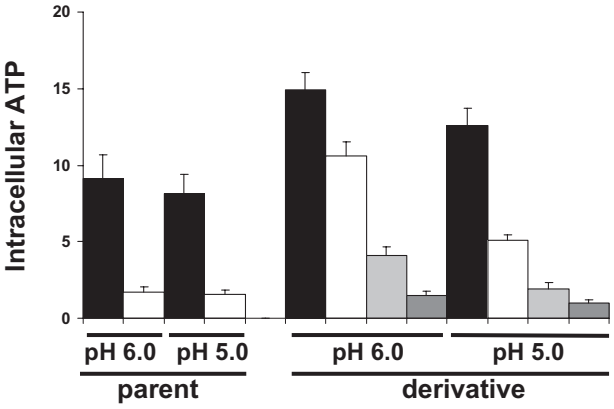

Fig. 5. Intracellular ATP content (nmol ATP mg^{-1} protein) of cells of the parent and the bile-resistant mutant grown at pH 6.0 or pH 5.0 in the absence (solid bars) or presence of 3 g l^{-1} bile salts (white bars), 6 g l^{-1} bile salts (light grey bars) or 10 g l^{-1} bile salts (dark grey bars). Error bars are standard deviations of at least three assays.

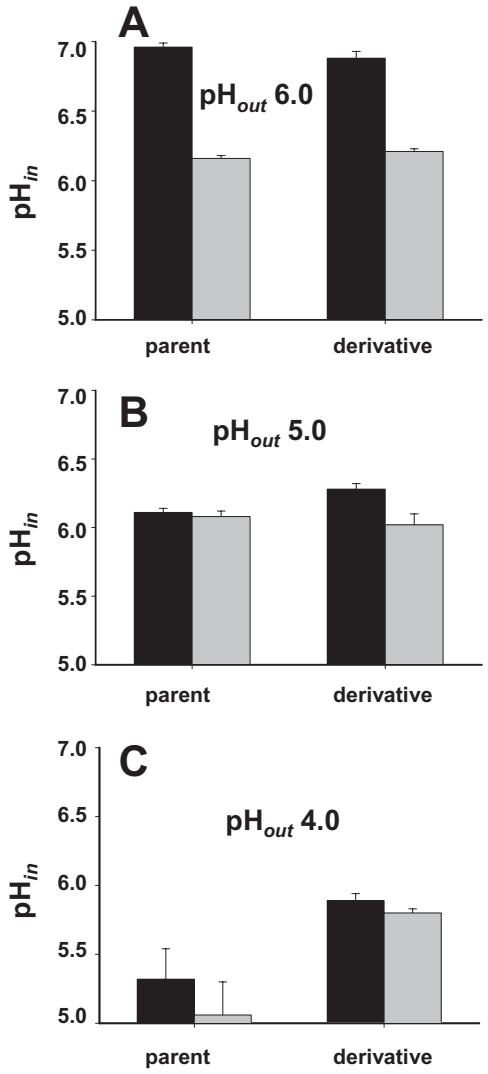

Fig. 6. Effect of the external pH (pH$_{out}$) on the intracellular pH (pH$_{in}$) of *Bifidobacterium animalis* IPLA 4549 and its mutant grown in the absence (solid bars) or presence (grey bars) of 3 g l^{-1} bile. The assays were carried out at external pH values of 6.0, 5.0 and 4.0.

H⁺-ATPase-deficient mutants were constructed in *E. coli* and *Lactococcus lactis*, which greatly facilitated the study of the physiological role of this enzyme (Jensen *et al*., 1993; Koebmann *et al*., 2000). However, for the time being, the lack of efficient transformation systems and the paucity of effective molecular tools (e.g. cloning and expression vectors and gene inactivation systems) strongly limits functional studies in *Bifidobacterium* (Ventura *et al*., 2004b). In the present work we have undertaken this challenge by analysing a bile-adapted mutant of a strain of *B. animalis* which has developed an increased pH tolerance after bile adaptation.

In a recent study we corroborated the identity of the strains *B. animalis* IPLA 4549 and its mutant with acquired resistance to bile *B. animalis* 4549dOx by partially sequencing the 16S rRNA gene and by analysing the restriction patterns from PCR fragments corresponding to the internal spacer region of the 16S-23S rRNA gene (Ruas-Madiedo *et al*., 2005). Furthermore, a recent proteomic analysis indicated a complete identity between the peptide mass fingerprints of 23 cytosolic proteins isolated from the parent and the bile-resistant mutant (B. Sánchez, M.-C. Champomier-Vergès, B. Stuer-Lauridsen, P. Ruas-Madiedo, *et al*., in preparation). This confirmed the identity of *B. animalis* 4549dOx as a bile-resistant mutant derived from *B. animalis* IPLA 4549.

Our previous evidences indicated that the viability of the bile-resistant mutant at pH 2.0 was significantly higher than the viability of the parent strain (Noriega *et al*., 2004). In the present work we corroborated that this is indeed the case. Relating to this, it is well known that the exposure to sublethal levels of a given stress allows cells to adapt and protect themselves against the subsequent exposure to normally lethal levels of other stresses (Begley *et al*., 2005). Exposing *Bifidobacterium* (Chung *et al*., 1999; Noriega *et al*., 2004; Saarela *et al*., 2004) and other Gram-positive microorganisms (Begley *et al*., 2002; Leverrier *et al*., 2003; Saarela *et al*., 2004) to either acid or bile increased tolerance to the other. Our data also pointed to a narrow relationship between acquisition of bile resistance and tolerance to acidic pH in *B. animalis*.

Anaerobic Gram-positive bacteria are strongly dependent on their membrane bound H⁺-F_1F_0-ATPase for generation of their proton motive force (Cotter and Hill, 2003). Recently, it was reported that F_1F_0-ATPase expression in *B. animalis* ssp. *lactis* DSM10140 was controlled at the transcriptional level by pH (Ventura *et al*., 2004a). Although a few literature reports have related the overproduction of some subunits of this enzyme with the exposure to bile (Leverrier *et al*., 2003; Bron *et al*., 2006), the link between the expression of this enzyme, its activity, and the exposure and/or adaptation of bacteria to bile has never been studied in detail until now. Interestingly, we found that the mutant has developed the ability to overproduce its F_1F_0-ATPase in the absence of bile at pH 5.0 to similar levels as when bile was present in the growth medium, an effect that was not observed in the parent strain. The F-type nature of the ATPase and the membrane-bound ATPase activity associated with this enzyme was confirmed by F_1F_0-ATPase effectors and especially by immunoblotting studies. These results indicated that the bile-adapted strain is able to respond to the stress promoted by acidic pH in a different way and more efficiently than its parent strain.

The response of enteric bacteria to bile is a complex physiological process which likely involves several mechanisms (Gunn, 2000; Begley *et al*., 2005; Sánchez *et al*., 2005). As bile renders the cell membranes more permeable to protons, they have been proposed to exert a pro-

tonophoric-like action (Zhao and Hirst, 1990; Amor *et al*., 2002). On the other hand, bile is mainly composed of weak acids (bile acids) that can diffuse through the lipid membranes under physiological conditions. Once entered into the cell they dissociate, increasing the intracellular proton pool (Kurdi *et al*., 2003). Furthermore, we have recently demonstrated that the acquisition of bile resistance by *B. animalis* ssp. *lactis* IPLA 4549 altered the fermentation pattern of carbohydrates, which theoretically improved the cellular ATP yield (Ruas-Madiedo *et al*., 2005). All these facts pointed to a regulation of intracellular pH and intracellular ATP content that could somehow be related to the ability of the bile-resistant mutant to survive under acidic conditions. To corroborate this assumption, we have measured the intracellular ATP content. In fact, the amount of ATP was always higher in the mutant than in the parent strain for a given bile concentration and/or acidic pH. In addition, the impact of bile and/or low pH on the reduction of intracellular ATP levels was much less pronounced in the mutant than in the parent strain. This was also directly related to the ability of the mutant to maintain the pH_{in} close to 6 when the pH_{out} was as low as 4.0, whereas the parent strain was not able to keep a pH_{in} higher than 5.3, independently on the presence of bile. Then, we correlated the increased ATP reserve with an enhanced ability of the mutant to maintain physiologically relevant internal pH under stress (pH or bile) conditions, although we can not rule out the involvement of other cellular processes such as ATP-dependent bile efflux pumps (Yokota *et al*., 2000; Sleator *et al*., 2005), or the requirement of this nucleotide for many of the ATP-dependent proteases or chaperones which are involved in stress response (Derre *et al*., 1999; Liu *et al*., 2005). Relating to this, recent results from our research group indicated a significant increase in bile salt hydrolase activity in the bile-adapted mutant (Noriega *et al*., 2006), being the enzyme responsible for this activity overproduced more than twofold as deduced by a proteome comparison of both strains (B. Sánchez, M.-C. Champomier-Vergès, B. Stuer-Lauridsen, P. Ruas-Madiedo, *et al*., in preparation). Hydrolysis may decrease the toxicity of conjugated bile salts (De Smet *et al*., 1995), indicating that other genes could also contribute to the observed bile- and pH-resistant phenotype.

In conclusion, a correlation between the intracellular ATP content, the regulation of the internal pH and the activity of the F_1F_0-ATPase in *B. animalis* in relation to its ability to adapt to bile was established. Our results suggest that the bile-adapted mutant of *B. animalis* become tolerant to low pH due to the action of the F_1F_0-ATPase, which compensates for the proton motive force-dissipating and internal pH-decreasing effects of bile by producing extra ATP that could be used to feed this enzyme, thus pumping the excess of protons from the cytoplasm to the external environment. This study establishes a new approach to investigate the capacity of bacteria to develop different strategies to cope with stress conditions in their environments.

Experimental procedures

Organisms and growth conditions

Bifidobacterium animalis ssp. *lactis* IPLA 4549 and its bile salt-resistant mutant *B. animalis* ssp. *lactis* 4549dOx (Noriega *et al*., 2004; Ruas-Madiedo *et al*., 2005) were used as model microorganisms throughout this study. The induced bile resistance phenotype was stable after 30 subcultures in the absence of bile (data not shown). Both strains were grown anaerobically at 37°C in MRS broth (Biokar Diagnostics, Beauvais, France) supplemented with 0.05% L-cysteine (w/v) (Sigma-Aldrich, St Louis, MO) (MRSC) in an anaerobic chamber (Mac500, Down Whitley Scientific, West Yorkshire, UK) under a 10% (v/v) H_2, 10% (v/v) CO_2 and 80% (v/v) N_2 atmosphere. The initial pH of the medium, when appropriate, was set to 5.0 and 6.0 with HCl, and bile salts (ox-bile extract LS55, Oxoid Limited, Hampshire, UK) were added at concentrations ranging between 3 g l^{-1} and 10 g l^{-1}.

The media were inoculated at 1% (v/v) with overnight cultures and then incubated anaerobically and monitored spectrophotometrically at 600 nm. Growth rates (μ) were estimated from the growth curve by fitting the data to the equation $N_t = N_0 \times e^{\mu \times t}$, in which N_t and N_0 are the cell densities at time t and time 0 respectively.

Tolerance to low pH in energized conditions

Amounts of 0.2 ml of overnight cultures were centrifuged, washed with NaCl 5 g l^{-1} and resuspended in 2.0 ml of an acid solution (NaCl 5 g l^{-1} adjusted to pH 2.0 with HCl, with or without glucose 25 mM) obtaining final counts of about 10^8 colony-forming units (cfu) ml^{-1}. Aliquots of 0.1 ml were taken at fixed time intervals after incubation at 37°C and viable cell counts (cfu) were determined on MRSC agar. Results were expressed as count decreases in cfu ml^{-1} with respect to the counts in a control solution (NaCl 5 g l^{-1}, pH 5.4) after incubation. Experiments were performed at least three times.

Isolation of membrane vesicles and immunodetection

Inside-Out membrane vesicles were obtained essentially as indicated in a previous work (Margolles *et al*., 2003) with minor modifications. Cells were grown in MRSC at pH 5.0 or 6.0 (pH adjusted with hydrochloride acid), with or without added bile salts (3 g l^{-1}), until the optical density at 600 nm (OD_{600}) reached values of 0.6 (after growth, the pH of the medium adjusted to pH 6.0 was about 5.7, and the pH of the medium adjusted to pH 5.0 remained unchanged, independently on the presence of bile). Then, cells were centrifuged at 2800 *g* at 4°C for 15 min and washed twice with 100 mM MES-potassium buffer. The pellet was resuspended in 4 ml of the same buffer supplemented with 10 mM $MgSO_4$, and treated with 10 mg ml^{-1} lysozyme and 50 units ml^{-1} mutanolysin. The suspension was incubated at 30°C for 4 h with constant stirring. Cells were broken by passage three times through a French pressure cell (20 000 p.s.i.) (SLM and

Aminco, Rochester, NY) and the suspension was incubated for 20 min at 30°C with 100 μg ml^{-1} DNase A (Sigma). Unbroken cells and cell debris were removed by two centrifugation steps at 13 000 *g* for 20 min at 4°C. Membrane vesicles were centrifuged at 125 000 *g* for 60 min at 4°C, resuspended in 50 mM MES-potassium buffer pH 7.0 plus 10% glycerol, frozen in liquid nitrogen and stored at −80°C until use.

Protein concentration was estimated using the BCA protein assay kit (Pierce, Rockford, IL), and amounts of 30 μg of protein were run on SDS-PAGE gels (10% acrylamide–0.27% bisacrylamide). The samples were then transferred to PVDF membranes (Amersham Biosciences, Buckinghamshire, UK), which were blocked with 5% non-fat dry milk in PBST (disodium phosphate 58 mM, sodium phosphate 170 mM, sodium chloride 68 mM, pH 7.3, plus 0.1% Tween-20, v/v) for 3 h at room temperature, incubated overnight at 4°C with primary antibodies (polyclonal antibodies against subunit β of *E. coli* ATP synthase, diluted 1:2000) containing 5% milk, washed extensively with PBST, and then incubated with the secondary antibody [peroxidase-conjugated goat anti-rabbit or anti-mouse IgG (Amersham Biosciences)] at 1:10 000 dilution in PBST with 5% milk. The blots were detected with an enhanced chemiluminescence kit (ECL, Lumigen™ TMA-6, Amersham Biosciences). The expression signal was estimated by densitometry using the software ImageMaster 2D Platinum (Amersham Biosciences).

ATPase assay

ATPase activity was calculated from the release of inorganic phosphate measured as described (Sakamoto *et al*., 2002). Inside-Out membrane vesicles (5 μg of membrane protein) were incubated at 37°C for 10 min in 50 mM MES-potassium buffer (pH 5.25) containing 5 mM $MgCl_2$. ATP (disodium salt, Amersham) was added at a final concentration of 300 μM to initiate the reaction. The reaction (total volume of 30 μl) was stopped after 5 min by immediately cooling the tubes on ice. Malachite green solution (150 μl of 0.034%) was added, and after 40 min the colour development was terminated by the addition of 22 μl of citric acid solution [34% (w/v)]. Immediately, the absorbance at 660 nm was measured. One unit of ATPase activity was defined as the release of 1 μmol of inorganic phosphate in 1 min. Calibration was performed by using a series of acetyl phosphate standards. For the determination of pH dependency of the ATPase activity, membranes were incubated for 60 min on ice in 50 mM MES-potassium buffer, adjusted to various pH values (4.0–6.5). To measure the effect of inhibitors on the ATPase activity, the membranes were pre-incubated with DCCD (Sigma; final concentration 0.2 mM), *ortho*-vanadate (Sigma; final concentration 0.2 mM) or nitrate (K_2NO_3; final concentration 25 mM) for 10 min at 37°C, and subsequently for 60 min on ice. The activity of membrane samples without any inhibitor was measured and used as control.

Measurement of intracellular ATP content

Cells were grown in MRSC at pH 5.0 or 6.0 and with or without bile salts (3 g l^{-1}, 6 g l^{-1} or 10 g l^{-1}) until OD_{600} of 0.6. To achieve cell-free extracts, the pellet was resuspended in 1 ml of lysis buffer (ATP Bioluminescence Assay kit HSII, Roche Applied Science, Mannheim, Germany) and cells were sonicated while cooling on ice using a CV17 sonicator (VibraCell, Sonics & Materials, Danbury, CT) and centrifuged to remove cell debris. The amount of ATP present in the cell lysates was measured by bioluminescence in accordance with the manufacturer's instructions. ATP standard solutions were used to convert arbitrary bioluminiscence units into molarity of ATP. Bioluminiscence was measured in a Cary Eclipse Fluorescence Spectophotometer (Varian, Palo Alto, CA) with 1 s integration time. Results are expressed as nmol ATP mg^{-1} protein.

Measurement of intracellular pH

Internal pH measurements were performed as described previously (Breeuwer *et al*., 1996), using the internally conjugated fluorescent pH probe carboxyfluorescein succinimidyl ester (cFSE; Molecular Probes, OR, USA). Briefly, cells were cultured until OD_{600} of 0.6, harvested and washed twice in CPK buffer (sodium citrate 50 mM, disodium phosphate 50 mM, potassium chloride 50 mM) at pH 7.0. Cells were resuspended in CPK buffer at different pH values (6.0, 5.0 and 4.0, adjusted with NaOH) in the absence or presence of bile (3 g l^{-1}), and incubated at 37°C for 30 min in the presence of the precursor probe carboxyfluorescein diacetate succinimidyl ester, washed twice, resuspended in CPK buffer at the corresponding pH and incubated for 15 min with 15 mM glucose to eliminate unbound probe. The cells were subsequently washed and resuspended in CPK buffer, pH 4.0–6.0, and placed on ice until pH measurements were carried out.

Internal pH values were calculated from the ratio of the fluorescent signal obtained at 490/440 nm. Previously, calibration curves were made for each strain (parent and mutant) in buffers with pH values between 4.0 and 7.0. The pH_{in} and pH_{out} were equilibrated by addition of valinomycin (1 μM) and nigericin (1 μM) and the ratios were determined as described previously.

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